CHAPTER 8

The Rhizosphere of Mycorrhizae

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I. Introduction

A. Concept of the Rhizosphere

1. The Problem in Perspective

In 1904, Hiltner introduced the term "rhizosphere" to mean the portion of the soil subject to the influence of the root systems of plants, and he noted that in this area there was greater microbial activity than in portions of the soil further away from the roots.

Since 1904, there have been numerous researches on the subject. Starkey (1929a,b) interpreted the influence of higher plants on the soil in a broader sense. According to him, this influence manifested itself by a diminution in the soil of certain mineral nutrients owing to root absorp-

tion, with the partial drying of the soil owing to removal of water by the roots, by an increase in the carbonates produced by roots secreting carbon dioxide, and by the growth of all microorganisms which feed on portions of disintegrating roots or their secretions. Some of these effects directly concern the soil population, others concern the soil population only indirectly by modifying the physical composition of the soil.

Studies have also been conducted on the influence exerted by rhizospheric organisms on plants. This influence is exerted through production of nutrients useful to plants, through decomposition of organic matter in the soil, and through the solvent action of carbon dioxide and other acids produced by those organisms, with transformation of nitrogen and sulfur compounds into readily available forms for which the higher plants compete. A variety of nutrients, minerals, and vitamins are found in the rhizosphere, leading to complex forms of competition and interaction.

From the very earliest investigations, the area of influence of the root has been one of the most important aspects in the study of rhizospheric microorganisms.

Gräf (1930) and Poschenrieder (1930) distinguished between the rhizosphere of the soil adhering to the roots and the rhizosphere of the surface of the roots themselves. The location of the microorganisms on the roots and around the root hairs was demonstrated by the contact slide method (Hulpoi, 1936; Isakova, 1938; Starkey, 1938; Linford, 1939).

To estimate the "rhizospheric effect," based on the number of microorganisms present, it is necessary to make a comparison with the number of microorganisms present in a portion of soil further from the roots. Comparisons with test soils are particularly important in showing a selective action of the roots on defined types or groups of microorganisms.

The number of microorganisms in the root zone is naturally influenced by many factors, such as the nature and age of the plant, soil type, moisture, soil reaction, and agricultural operations, and it is not always possible to determine which of these is responsible for the observed effects. Soil operations, for instance, may stimulate the growth of the plant, which, in turn, might cause an increase in the number of microorganisms on the root surface.

Numerous quantitative comparisons of rhizospheric microorganisms in different plants, grown under identical environmental conditions, have been conducted. However, these data cannot always be compared due to the fact that, as a rule, they are obtained from plants of the same age but assuredly not at the same stage of development, because of their different rates of growth.

Starkey (1929c) noted that the roots of alfalfa (*Medicago sativa*) stimulated fungi only slightly, whereas eggplant (*Solanum molongena*)

had a more appreciable effect leading to an increase in the number of actinomycetes and bacteria, while beetroot and corn were less active. These phenomena are even more evident in forests. In fact, in a young forest of relatively undeveloped specimens, the influence of the trees on the microbial flora of the soil is limited, since there is a modest production of root exudates, owing to the scanty root development. These conditions change visibly, however, with tree growth, accompanied by a progressive increase in the root systems which gradually invade the available soil, intertwining and influencing practically all the microbial flora of the soil environment.

In general, the majority of the plants do not exert any appreciable effect during the first stages of growth, and usually the maximum effect appears after the plant has reached a moderate stage of development, at the time of flowering or at the start of the senescing process. The rhizospheric effect, therefore, varies during the plant's growth. The germination of the seed also coincides with the appearance of new soil microorganisms, i.e., those which constitute the spermosphere. The phenomenon also involves liberation of rapidly degradable substances which form the sheath of the seed and stimulate microbial multiplication. Some investigators consider that the microorganisms of the spermosphere take an active part in colonizing the roots (Tardieux *et al.*, 1961), while others make a contrary claim that root colonization is solely the work of soil microorganisms (Parkinson *et al.*, 1963).

In many cases the greatest rhizospheric effect corresponds to flowering or the period preceding this stage (Vančura and Hovadik, 1965). Views of the subject are, however, still at variance, and, according to some investigators, the greatest rhizospheric effect occurs at other stages in the plant's growth (Rivière, 1959). When roots age, the rhizospheric effect is gradually masked by proliferation of microorganisms which intervene in the decomposition of the dead root tissues.

The rhizospheric effect also varies greatly from plant to plant. In forest trees it proceeds regularly and in the majority of cases in proportion to growth. In herbaceous plants, on the other hand, the greatest effect can be characterized by a particular stage of development. Potatoes (*Solanum tuberosum*) and oats (*Avena sativa*) display a rapid increase in the number of rhizospheric microorganisms after the first stage of growth and a progressive decline after maturity and death. Biennial plants show a marked effect towards the later stages of their development.

Gräf (1930), besides noting a distinction between the rhizosphere of the root surface and that of the soil adhering to the root, also noted through weekly checks that a considerable increase in the number of microorganisms on the root surfaces of cereals during the growth period was fol-

lowed by a slight decrease during the plant's maturation period, and a considerable increase after harvesting. He also noted that the increase in the total number of microorganisms was small in the case of wheat (*Triticum vulgare*) and much greater with legumes and turnips (*Brassica*).

In a number of annual plants Thom and Humfeld (1932) found greater stimulation in the number of bacteria rather than the fungi and the actinomycetes. However, they indicated that this phenomenon appeared to be linked primarily to the nature of the soil. In 1934, Krasilnikov noted that during growth the rhizosphere of groundnuts (*Arachis hypogaea*) hosted 10 to 100 times the number of microorganisms than that encountered in soil distant from the roots. This condition continued until the flowering stage. Toward maturity the number of bacteria decreased, whereas the number of fungi and actinomycetes started to increase.

Obraztzova (1935) pointed out a remarkable rhizospheric effect in the roots of tea (*Camellia theifera*) that was linked to the age of the plant. Twice the number of microorganisms were observed in 2-year-old plants than those present in the rhizosphere of an old plant. In many leguminous plants, the number and activity of the bacterial population of the rhizosphere varies considerably with species and stage of plant development.

Sabinin and Minina (1932) observed an extraordinary example of the influence of root systems on terrestrial microflora in desert soil. Microbial life was present only in the root systems of plants. Clark (1940a), studying the influence of fertilizers on rhizospheric populations, noted a greater number of rhizospheric microorganisms in a treated soil sample than in an untreated soil sample. This difference was also evident in relation to the nonrhizospheric microflora present in a soil that had been given massive quantities of fertilizer. In further studies, he compared the rhizosphere of cotton (*Gossypium*) with that of herbaceous plants and found a greater number of bacteria in the former.

Adati (1939) performed extensive work on the number of bacteria, actinomycetes, and fungi in the rhizospheres of different plants growing in sandy, claylike soils of various types and in humus soils. He made extensive comparisons with soil taken from around roots and some distance away from the roots. Many organisms were found in the soil directly adjacent to the roots, with the number inversely proportional to the distance from the roots. The bacteria were markedly stimulated, the fungi less stimulated, and, in general, the actinomycetes even less stimulated.

Timonin (1940), comparing the rhizospheres of cotton (*Gossypium*), oats, alfalfa, and peas (*Pisum sativum*), found that the bacteria and actinomycetes together were from 7 to 71 times more numerous and the

fungi from 0.75 to 3.1 times more numerous in the rhizosphere than in the control soil. The second group of organisms was considerably more abundant in the rhizosphere of oats and barley (*Hordeum vulgare*), while the first was more abundant in that of alfalfa. In general, an increase in the rhizospheric effect was noted during the growth of the plants.

Zukovskaya (1941), studying the rhizospheres of potato, flax (*Linum usitatissimum*), and clover (*Trifolium pratense*), showed that each plant had the capacity to stimulate individually a specific microflora. Berezova (1941) identified different types of organisms on flax plants, associated with the plant at different growth stages. From both the quantitative and the qualitative standpoint, he distinguished the rhizosphere of the soil next to the roots, populated by aerobic bacteria, fungi, and actinomycetes, and the rhizosphere of the root surface with the denitrifying bacteria of the *Agrobacterium radiobacter* group and *Bacillus macerans*.

The large number of microorganisms normally present around plant roots presupposes intensive metabolic activity in this region. This activity has been measured in terms of CO_2 production and naturally is much greater in soils covered by vegetation than in plantless soils. The production of CO_2 stems mainly from two sources: the plant, as a product of respiration of the root cells, and the microbial activity near the roots, which causes decomposition of root secretions. Which of these two sources provides a more abundant quantity of CO_2 depends upon the general environmental conditions, the stage of growth, and the nature of the plant. According to Waksman (1931), 45% of this gas can be produced by microbial activity.

Starkey (1929c) observed that small amounts of CO_2 were produced in the first stages of plant growth, followed by the greatest production at the stage of fastest growth, and finally a decrease during the mature stage. In further studies, Starkey (1931) noted greater amounts of CO_2 in soils taken from the regions of major root development than in soils taken from regions more distant from the roots, thereby again showing a correlation between intensive CO_2 production and abundance of microbes in the soil.

Stille (1938) compared production of CO_2 by mustard roots (*Brassica nigra*) grown in sterile sand and in sand inoculated with a soil suspension. He found greatest CO_2 production in the inoculated sand. Barker and Broyer (1942), checking the production of CO_2 by pumpkin (*Cucurbita maxima*) roots with the addition of manganese, obtained 0.279 μl in a sterile aerobic culture and 0.549 μl in a nonsterile culture, i.e., about 50% of the CO_2 was produced by the roots. Under anaerobic

conditions, the sterile system produced 0.108 μl and the nonsterile system 0.289 μl, i.e., about 37% was produced by the roots.

2. *Qualitative Aspects of the Rhizosphere*

Numerous studies have been made of the qualitative influence exerted by higher plants on the microflora of the soil, especially on bacteria. Investigations have been conducted along two main lines: the study of bacterial groups using selective media and the study of individual bacterial types from morphological and physiological viewpoints. An attempt will now be made to provide an idea of the groups of microorganisms living in the rhizosphere according to a qualitative criterion which stresses the specific function of each group.

Many writers, rather than dwelling on quantitative investigations of a sterile root environment, have considered it important to understand the interactions between plant roots and soil microflora in order to estimate the physiological activity and nutritional needs of the organisms encountered on the root surfaces. The production of CO_2 by the soil has often been interpreted as an index of microbial activity, in view of the greater production of this gas in the soil adjacent to the roots. This activity is related to the abundance of organisms in the soil adjacent to the roots and to the availability of organic substances to be broken down. Thom (1935) observed that many of the organisms associated with the rhizosphere belong to the species active in the decomposition of organic matter. Clark (1940b) noted that the *Pseudomonas* bacteria present in the soil were different from those associated with roots. The majority of those isolated from the soil were unable to utilize saccharose or to hydrolyze amides, whereas those isolated from the rhizosphere fermented the more complex sugars.

By using slides and isolating material on nonselective agar, Lochhead (1940) also found greater microbial activity in the rhizosphere than in the soil distant from the roots. He found a higher percentage of forms in the rhizosphere which grew well on nutrient agar and dissolved the substrate and forms which cause acid or alkaline reactions in the presence of dextrose. He also observed that a large percentage of the bacteria belonged to the motile, chromogenous type. Relatively abundant chromogenous forms were found in the rhizosphere of tobacco (*Nicotiana tabacum*) plants. Lochhead also observed a physiologically more active microflora in the rhizosphere of certain varieties of flax and tobacco.

A qualitative study of soil bacteria, using the technique of Taylor and Lochhead (1938), was carried out by West and Lochhead (1940). They investigated the nutritional needs of rhizospheric organisms isolated with the use of a nonselective cultural medium (soil extract) by adding

TABLE I

COMPARISON BETWEEN THE NUMBERS OF CULTURES WITH POSITIVE GROWTH IN EACH MEDIUM IN *Pinus radiata*, *Pinus pinea*, *Populus euramericana*, AND *Eucalyptus camaldulensis*

Nutritional groups	Rhizosphere (%)				Soil control (%)			
	P. radiata	*P. pinea*	*Populus*	*Eucalyptus*	*P. radiata*	*P. pinea*	*Populus*	*Eucalyptus*
Good growth in basal medium	11	18.6	0	2	7	12.5	0	2
Require amino acids	17	31.8	21	18	2	10.0	20	3
Require growth factors	3	4.3	14	8	2	3.7	3	2
Require amino acids + growth factors	22	8.7	9	14	0	12.5	11	9
Require yeast extract	19	31.8	19	16	24	16.2	14	67
Require soil extract	3	1.1	0	4	9	20.0	2	0
Require yeast + soil extract	11	2.1	11	9	20	23.7	27	8
No growth	14	1.1	27	29	36	1.2	23	9

amino acids, growth factors, yeast extract, and soil extract to an inorganic medium, and testing the development of individual types in these media. They identified the nutritional requirements of a representative group of rhizosphere organisms. It was thus possible to ascertain that the roots of a number of plants favored the development of bacteria which needed thiamin, biotin, and amino acids for growth and microorganisms capable of synthesizing the essential growth substances on the simple basal medium only. This microbial distribution was typical for a number of plants.

West and Lochhead (1940) adopted the bacterial balance index (BBI) to identify the rhizospheric effect. This index, calculated by assigning a negative value to the percentage of bacteria growing abundantly in a simple inorganic medium and a positive value to organisms which needed amino acids and growth factors, provided interesting data on distribution of the rhizospheric flora.

A more extensive differentiation of nutritional groups of bacteria was proposed by Lochhead and Chase in 1943. The method consists of determining the development that can be noted in seven cultural media of increasing complexity. Using this method, Lochhead and Thexton (1947) and others ascertained the presence of bacteria able to synthesize growth factors and use amino acids in the rhizosphere of a number of plants. Similar results were later obtained by Rambelli (1962, 1963, 1965, 1966), who studied the rhizosphere of the mycorrhizae of *Eucalyptus camaldulensis*, *Populus euramericana*, *Pinus pinea*, and *P. radiata*. He obtained good utilization of amino acids in the mycorrhizosphere of the trees studied. With respect to synthesis of growth factors, however, positive results were noted in the rhizosphere of *Populus* and *Eucalyptus*, while the phenomenon appeared uncertain in that of *P. radiata* and *P. pinea*. These results, which indicate some degree of similarity in the physiological activity of the rhizosphere of *P. radiata* and *P. pinea*, are also borne out by checking the behavior of the bacterial types with respect to amino acid and growth factor requirements (Tables I–III). This phenomenon should cause no surprise because, whereas *Populus* and *Eucalyptus* formed mycorrhizae with systematically distant symbionts, *P. radiata* and *P. pinea* both formed mycorrhizae with *Boletus granulatus*. Considering that the rhizospheric microflora studied was isolated from the surface of the fungoid cover, it is logical to assume that this microflora undergoes similar stimuli in the last two plants.

In 1943, Lochhead and Chase also observed that filtered bacterial cultures, grown in a simple inorganic medium, gave a greater stimulus to the organisms which needed amino acids, whereas they exerted an antagonistic action on bacteria which required growth factors. This induced

TABLE II

AMINO ACIDS IN THE NUTRITION OF RHIZOSPHERE AND CONTROL BACTERIA. COMPARISON OF *Pinus radiata*, *Pinus pinea*, *Populus euramericana*, AND *Eucalyptus camaldulensis*

Amino acids	Rhizosphere (%)				Soil control (%)			
	P. radiata	*P. pinea*	*Populus*	*Eucalyptus*	*P. radiata*	*P. pinea*	*Populus*	*Eucalyptus*
No growth or submaximum growth in basal medium								
Maximum growth with amino acids	17	24	19	18	2	8	20	4
Maximum growth with cysteine	0	4	7	11	2	0	2	2
No growth in basal medium								
Growth (maximum or submaximum) with amino acids	19	10	15	14	13	34	23	8
Growth (maximum or submaximum) with cysteine	8	4	9	11	13	10	17	4
Submaximum growth in basal medium								
Stimulated by amino acids	19	45	32	35	13	18	35	40
Stimulated by cysteine	3	14	21	28	4	5	15	20
Submaximum growth in growth factor medium[a]								
Stimulated by amino acids	50	44	28	33	16	30	38	37

[a] See Lockhead and Texton (1947) for nomenclature.

TABLE III

GROWTH FACTORS IN THE NUTRITION OF RHIZOSPHERE AND CONTROL BACTERIA. COMPARISON OF *Pinus radiata*, *Pinus pinea*, *Populus euramericana*, AND *Eucalyptus camaldulensis*

Growth factors	Rhizosphere (%)				Soil control (%)			
	P. radiata	*P. pinea*	*Populus*	*Eucalyptus*	*P. radiata*	*P. pinea*	*Populus*	*Eucalyptus*
No growth or submaximum growth in basal medium or A.[a]								
Maximum growth with growth factors	3	2	14	28	2	1	3	4
Maximum growth, growth factors + amino acids	25	8	16	64	2	11	13	15
No growth in basal medium or A.[a]								
Growth (maximum or submaximum) with growth factors	11	0	12	44	2	0	2	7
Growth (maximum or submaximum) with growth factors + amino acids	11	0	16	60	2	1	5	8
Submaximum growth in basal medium								
Stimulated by growth factors	6	16	35	41	16	11	17	29
Submaximum growth on A.[a]								
Stimulated by growth factors	22	16	22	15	7	25	5	29

[a] See Lockhead and Thexton (1947) for nomenclature.

interactions in the rhizospheric microflora, which accompanied the influence exerted by the root exudates and decomposition of the root fragments.

Another important character of the rhizosphere (Wallace, 1947) concerns partial suppression of bacteria dependent on more complex substances contained in the soil extract. The investigations, carried out on herbaceous plants, were confirmed by Rambelli (1962, 1963, 1965, 1966) studying the rhizosphere of *Eucalyptus*, *Pinus pinea*, *Populus*, and *Pinus radiata*.

Wallace and Lochhead (1950), while studying the amino acid requirements of rhizospheric bacteria of a number of herbaceous plants, checked the development of strains in a medium containing 23 amino acids. The amino acid group containing sulfur (cysteine, methionine, and taurine) proved of special significance because it was indispensable for development of some microbial groups. Many studies, on organisms able to utilize this group of amino acids, have brought to light the importance of methionine in microbial metabolism.

Krasilnikov and Koreniako (1946) observed that certain species of *Pseudomonas* and *Achromobacter*, present in the rhizosphere of clover (*Trifolium pratense*), strongly influenced development of bacterial root nodules. While a number of species stimulate growth of the bacterial nodules, others inhibit it, and still others show no effect on it whatever.

It is generally admitted that legumes exert a very marked stimulatory effect on *Rhizobium*. By means of slide cultures, the existence was ascertained of an abundant microbial population in the rhizosphere of this plant (Nutman, 1957). In the rhizosphere of the legume *Astragalus* sp. the quantitative rhizosphere soil ratio varies between 3000 and 1000 for *Rhizobium*, but drops to between 100 and 400 for the overall microflora. There is therefore a preferential stimulation of *Rhizobium* which represents 1–10% of the total rhizospheric micropopulation.

Vančura *et al.* (1965) studied the rhizospheric effect on *Azotobacter* in Egyptian soils in which this microorganism is very abundant. The rhizospheric effect does not vary essentially from the description of this phenomenon in scientific literature, although the number of *Azotobacter* colonies in the rhizosphere of a number of plants may increase up to 10^7/gm of soil.

Investigations have also been carried out on the distribution of microorganisms belonging to the genus *Azotobacter* in the rhizosphere of other plants. Nevertheless, despite the interest which these microorganisms hold from a theoretical and a practical point of view, there is surprisingly little information of a quantitative nature concerning their natural participation in the rhizosphere of plants. Beijerinck, as long ago as 1888,

observed a greater number of *Azotobacter* organisms in a legume root soil than in a nonlegume root soil. Kostychev *et al.* (1926) found an accumulation of *Azotobacter* in the vicinity of tobacco roots. Comparing rhizospheric soil and nonrhizospheric soil, Gräf (1930) concluded that the most intensive nitrogen fixation by *Azotobacter* and by *Bacillus amylobacter* occurred in the rhizosphere.

In a study on the microflora of the root systems of 31 families of plants, Rokitzkaya (1932) observed *Azotobacter* in all except members of the *Pinaceae*, *Betulaceae*, and *Fagaceae*. The relative intensity of development of *Azotobacter* was at its maximum in members of the *Polygonaceae*, *Rosaceae*, *Pomaceae*, and others.

Undoubtedly many plants (*Pinaceae*, *Betulaceae*, *Fagaceae*, etc.) either have no effect on *Azotobacter*, or have no significant effect (Katznelson and Strzelczyk, 1961). Others, such as clover and vetch (*Vicia sativa*), however, seem capable of stimulating growth of *Azotobacter* (Vančura *et al.*, 1965); while others, such as *Chelidonium* sp. and *Ephedra distachya* (Daste, 1956), inhibit it. Hence, extensive research shows that a single plant could have, according to prevailing ecological conditions, an unfavorable or favorable effect, or no effect on *Azotobacter* development.

According to many investigators, *Pseudomonas* is frequently stimulated by *Arthrobacter*. This can be explained by the capacity of the former to produce substances inhibiting the development of other microorganisms. Chan and Katznelson (1961) found that different species of the genus *Pseudomonas* were not stimulated to the same degree in the different plants.

Using suitable media and microscopic observation, it was shown that the filamentous forms are abundant on the root surface. These are bacteria (*Corynebacterium*) and actinomycetes (*Mycobacterium* and *Nocardia*). *Clostridium* is frequently found in the rhizospheres of lupin (*Lupinus albus*), peas, poppies (*Papaver somniferum*), and radishes (*Raphanus sativus*) (Strzelczyk, 1958). The examples quoted show that it is not possible to detect a generalized behavior pattern in composition of the rhizospheric microflora.

In a broad sense, in the rhizosphere various spore-forming and nonspore-forming bacteria, such as *Bacillus mycoides and B. fluorescens,* play an active part in breaking down protein molecules and the simplest nitrogen compounds to liberate ammonia. Gräf (1930) noted that decomposition of peptone and the production of ammonia occurred abundantly in specimens taken from the root surface, increasing in intensity from May through July and dropping slightly in August. Rokitzskaya (1932) demonstrated the presence of an active ammonifying microflora in the root zone of 178 different plants. Krasilnikov *et al.* (1936) established

that the predominant forms in the rhizosphere were usually non-spore-forming bacteria with pronounced ammonifying activity. Lochhead (1940) observed forms that dissolved gelatin, and Timonin (1946) noted that forms which hydrolyzed casein were more numerous in the rhizosphere of various plants than in control soils.

Regarding ammonification, Rambelli (1966), while studying the development of bacteria isolated from the rhizosphere of *P. radiata* mycorrhizae in three different geographic areas of Italy, found a decidedly greater need for amino acids than those present in the control.

Very little research has been carried out on nitrifying bacteria in the rhizosphere. Many comparisons of rhizospheric and nonrhizospheric soil samples have shown the positive influence of plants in the first stage of growth and, in contrast, microbial inhibition in subsequent stages.

Starkey (1929c) compared the accumulation of nitrates in cultivated and noncultivated soil, and, in general, observed an increase in nitrates in the latter. Starkey (1931) showed that nitrification is favored in soils characterized by intense root development. He thus considered it reasonable that the soil constituents required by nitrifying bacteria were present in relative abundance in the vicinity of the roots. Truffaut and Vladykov (1930) isolated strains of *Nitrosomonas* from the rhizosphere of wheat, and Rokitzkaya (1932) observed nitrification of the root zone in all the plants examined. Rovira (1956) studied the effect of root exudates on terrestrial microflora by adding them to the soil. He obtained an increase in the number of gram-negative microorganisms, whereas nitrification and oxygen absorption only increased if glucose or peptone was added. Molina and Rovira (1964) confirmed the stimulation of *Nitrosomonas* and *Nitrobacter* by corn (*Zea mays*) and alfalfa roots. According to Rambelli (1970), nitrite- and nitrate-forming flora are stimulated in the mycorrhizae of *P. radiata.*

Investigations on the denitrifying group are more numerous. Chalvignac (1958), Gräff (1930), and others observed considerable denitrification of the root surface of certain herbaceous plants. Denitrifying organisms have been found in great abundance on the roots of wheat in fertilized soil, but not on alfalfa roots in fertilized soil. Investigations into the presence of denitrifying organisms in the rhizosphere of *P. radiata* (Rambelli, 1970) have shown a reduced number of denitrifiers in the rhizosphere of adult plants, but a number in excess of the control in seedlings grown in plastic pots.

Several investigators have shown that cellulose-decomposing bacteria are present in the rhizosphere of many plants. Krasilnikov (1936) described a marked increase of cellulose-decomposing bacteria in the rhizosphere of wheat, maize, sunflower (*Helianthus annuus*), and soybeans

(*Glycine soja*). He observed that as the plants grew there was an increase in the total number of anaerobic bacteria and organisms which decompose cellulose in the rhizosphere of sunflowers and soybeans. Some investigators claim that these plants take part in the decomposition of dead root fragments and that the products of the process are immediately metabolized by other microorganisms. Katznelson (1946) found numerous aerobic cellulose-decomposing bacteria in the rhizosphere of mangels (*Beta vulgaris* v. *macrorhiza*) grown in fertilized soil, but they occurred far more abundantly in that of mangels grown in unfertilized soil.

Investigations have also been conducted on the rhizospheric microflora capable of oxidizing manganese. This group of organisms has been studied especially in relation to plant diseases caused by manganese deficiency. Timonin (1946) observed a large number of microorganisms capable of oxidizing manganese in the rhizosphere of a variety of oats susceptible to diseases. Such a microbial population, on the other hand, is relatively scanty in the rhizosphere of resistant varieties.

Anaerobic microorganisms have been surprisingly neglected by many investigators working on the rhizosphere. In 1903, Velich found a great abundance of *Clostridium gelatinosum* in the soil close to the roots and the root surface of beet (*Beta vulgaris*), mangels, barley, rye, oats, and wheat. Rokitzkaya (1932) demonstrated the presence of *Clostridium* in the root zone of 31 plant families. The intensity of growth of this microorganism was greatest in the *Chenopodiaceae, Solonaceae,* and *Valerianaceae* and least in the *Pinaceae, Betulaceae,* and *Fagaceae* (the same families that do not possess *Azotobacter* in the rhizosphere). Katznelson (1946) observed a remarkable increase in the number of anaerobic forms on the root surface of mangels grown in both fertilized and nonfertilized soils. Here, too, an anaerobic microbial population developed in the rhizosphere.

3. *Nitrogen Fixation*[1]

In 1967, Foster and Marks, with the help of an electron microscope, identified bacterial cells in the fungal cover of mycorrhizae of *P. radiata* and suggested that these may be associated with nitrogen fixation. Rambelli (1970), studying the rhizosphere of mycorrhizae of *P. radiata*, isolated, in a pure culture, a bacterial strain that developed well in a liquid mineral medium without any nitrogen and in an environment of air filtered through a 20% sulfuric acid solution. This microorganism seems able to fix small quantities of atmospheric nitrogen. It probably obtains

[1] See Chapter 5.

its nutrition from the fungal symbiont (apart from the possible fixation of atmospheric nitrogen). This bacterium showed rapid and vigorous growth when cultivated in a nitrogen-free Hagem liquid medium (pH 7.3) in an atmosphere devoid of ammoniated nitrogen vapors and with 0.01% of the metabolites extracted from the fungal symbiont of *P. radiata.* This observation probably has some significance, especially in the light of the supposed fixation of atmospheric nitrogen by bacteria within the fungal cover. The phenomenon is an extension of the mycorrhizal concept since this microorganism would be adjacent, for symbiotic purposes, to two other elements characteristic of mycorrhizal symbiosis, the plant and the fungus.

Subsequently, Rambelli (1970) investigated the distribution of the nitrogen-fixing microflora of mycorrhizal and nonmycorrhizal seedlings of *Pinus radiata.* He carried out the following experiment in the nursery of the Rome Agriculture and Forestry Experimentation Center.

Carefully washed siliceous river sand was placed in two wooden boxes $1 \times 1 \times 0.60$ m in size. The sand was washed primarily to remove as much inorganic matter as possible. *Pinus radiata* seeds were sown in one of the boxes. After 8 months growth the seedlings were very small, chlorotic, and lacking in vigor. At that time a certain number of *P. radiata* seedlings of the same age, i.e., 8 months old, grown in plastic pots with soil rich in mycorrhizal organisms, were taken from the Agriculture and Forestry Experimentation Center nursery. The root systems of the latter seedlings were well furnished with mycorrhizae of the chestnut-brown form. These seedlings were considerably better developed. They were given a careful preliminary washing to free their root systems from adhering soil fragments and were then subjected to prolonged washing in running water, after brushing away all traces of mineral fragments or organic substances adhering to the root system. The root portions which could not be washed carefully were eliminated. After this treatment, the seedlings were planted in a second box similar to the first containing washed sand. No fertilizer was added to either box. They were watered periodically in line with ordinary cultural practices.

One month after planting, the seedlings in the second box had taken root and growth had started, although it was not as vigorous as that of plants in plastic pots or in the open field. The seedlings in the first box, on the other hand, were growing slowly, and they exhibited distinct symptoms of chlorosis. After another 3 months, i.e., 12 months after sowing, the differences in size were even more obvious. It then seemed of interest to examine the mycorrhizal condition of both boxes. A few seedlings from each box were sampled and their roots were washed very carefully. The plants from the first box showed a completely non-

mycorrhizal root system, the plants in the other box had abundant, well developed, chestnut-brown mycorrhizae. The rhizospheric microflora of the root tips of both boxes was examined as before, by washing a certain number of root tips six times in sterile distilled water. The water from the sixth washing was used for isolation. Twenty-seven bacterial strains were isolated from the nonmycorrhizal tips, and 40 strains from the mycorrhizal tips of the second box. In the first batch, 24 of the strains were able to develop in a mineral medium without nitrogen and in the presence of air filtered through a solution of sulfuric acid. All 40 bacterial strains from mycorrhizal root tips in the second box were able to develop in a liquid mineral medium without nitrogen and in the presence of air filtered through a sulfuric acid solution (Table IV).

4. Succession of Rhizospheric Microflora

Direct microscopic examination has revealed the presence of a considerable population of actinomycetes on the root surface where these microorganisms are most likely to play a prominent role in the relationships of the rhizosphere. Quantitative evaluation techniques generally show a positive rhizospheric stimulation for fungi, but, except for some exceptions, this aspect is less obvious for bacteria. Rovira (1965b) pointed out in this respect that if the rhizospheric effect is considered in terms of the cellular mass and not the number of cells and propagules, the relationship is probably as distinct for bacteria as it is for fungi.

Harley and Waid's techniques (1955) have led to much progress in the study of the fungal rhizosphere. Their method makes it possible to show a clear succession of fungi on the roots in relation to root development (Parkinson *et al.*, 1963). The initial disorderly colonization effected by a fairly large number of fungal species is superseded by stable

TABLE IV

GROWTH OF BACTERIAL STRAINS (ISOLATED FROM MYCORRHIZAL AND NONMYCORRHIZAL TIPS OF *P. radiata* GROWN IN WASHED SAND) IN A NITROGEN-FIXING MEDIUM AND IN AIR FILTERED THROUGH 20% SULFURIC ACID SOLUTION

Bacterial strains isolated from					
Mycorrhizal tips			Nonmycorrhizal tips		
No. of strains	N-fixing agar	N-fixing solution	No. of strains	N-fixing agar	N-fixing solution
40	+38 −2	+40 −0	27	+27 −0	+24 −3

colonization of specialized forms closely dependent on both the plant and soil. As the roots age, the cortical tissue is colonized more and more deeply by the fungi.

Parkinson and Thomas (1969), working with dwarf bean (*Phaseolus vulgaris*) plants, studied changes in the fungal flora during colonization of roots at different stages of growth of the plant. In general, there was weak microbial stimulation in the first stages of growth which increased in parallel with vegetative growth of the plant and lessened considerably during senescence. Respiration studies on samples of rhizospheric and nonrhizospheric soil indicated greater activity in the former. The degree of maximum microbial stimulation appears to correspond to the plant's maximum vegetative activity.

Timonin (1964) studied the rhizosphere of healthy and diseased seedlings of *Pinus contorta,* and showed a distinct difference in rhizospheric organisms present. Isolations from the rhizosphere of plants and seeds of both healthy and diseased plants have shown that *Aspergillus, Phoma, Pythium, Rhizoctonia,* and *Rhizopus* occurred only in the former, and *Alternaria, Cephalosporium, Metarrhizium, Spicaria,* and *Tilachlidium* in the latter. Rambelli (1962), studying the rhizosphere of the mycorrhizae of *Eucalyptus camaldulensis,* identified the following fungi that were absent in the control: *Penicillium implicatum, P. sclerotiorum, P. cyclopium, P. decumbens, P. fellutanum, Aspergillus flavipes, Tieghemella spinosa,* and *Trichoderma koningi.* Rambelli (1965) subsequently observed a smaller number of fungi in the rhizosphere of *Populus euramericana* than in that of the control, and he considered that rhizospheric stimulation is able to select the fungal microflora, enabling only given microbial forms to develop. These findings however do not agree with those made by studying the fungal rhizosphere of mycorrhizae of *Pinus radiata* in three geographically different stations in Italy. In the latter investigations, Rambelli (1966) constantly showed a distinct dominance of the fungal flora in the rhizosphere.

Investigations on the qualitative distribution of the fungal population, however, are relatively scarce. Thom and Humfeld (1932) refer to the colonization of wheat roots in acid soils by *Trichoderma,* while the roots in the alkaline soils appeared associated, above all, with *Penicillium* of the biverticillata series (*P. luteum*). According to Timonin (1940), there is a significant difference in the rhizospheric and nonrhizospheric fungal population of many plants. He provided information in 1941 on the isolation of ten fungi present only in the rhizosphere of flax. Certain genera such as *Alternaria, Aspergillus, Cephalosporium, Fusarium, Helminthosporium,* and *Verticillium* were numerically more abundant in the rhizosphere of varieties of flax sensitive to root rot, while others,

such as *Mucor, Cladosporium, Hymenula, Penicillium, Scolecobasidium,* and *Trichoderma,* were more abundant in the rhizosphere of resistant varieties.

Katznelson and Richardson (1943), in a study on the qualitative differences of the fungi of the rhizosphere of the tomato, identified a selective action by the roots, especially regarding an unidentified component of the fungal population. The rhizosphere of plants in soil treated with acids was characterized by forms of *Penicillium* (26%), *Verticillium* (28%), and *Cladosporium* (4%). The control soil, on the other hand, contained *Aspergillus* (6%), *Trichoderma* (15%), *Oothecium* (16%), an unidentified fungus (24%), and other forms such as *Mucor* and *Fusarium.* Their later researches yielded results which indicate a selective action by roots on soil fungi. The remaining groups of microorganisms of the soil, such as protozoa and algae, have not been examined from the qualitative standpoint.

5. *Interrelationships of Rhizosphere Microorganisms*

Within the rhizosphere, the phenomena of synergism, satellitism, and antagonism are particularly intense. Since these interactions play an important role in the balance between saprophytes and parasitic microorganisms, their study is open to noteworthy applications in biological control of infectious plant diseases caused by bacteria, fungi, and actinomycetes.

Numerous investigations have shown rhizospheric stimulation of a number of microbial associations, generally microorganisms which synthesize and use vitamins and various growth factors. These associations are of some use to the plant, if they are likely to favor its growth directly or indirectly. Synergistic associations can also be formed among phytopathogenic microorganisms which thereby increase their virulence toward the host, e.g., root rot (caused by *Thielaviopsis basicola*) of a number of varieties of tobacco is assisted by rhizospheric bacteria which permit development of the fungal pathogen. In contrast, in species resistant to root rot, there is a rhizospheric stimulation of bacteria which inhibit the development of the parasitic fungus (Strzelczyk, 1966).

Antagonism can also develop among rhizospheric microorganisms. Such phenomena become manifest, with differing intensity, in rhizospheric and nonrhizospheric soils. According to Chan and Katznelson (1961), *Pseudomonas* sp., a typical microorganism of the rhizosphere, and *Arthrobacter globiformis,* a typical microorganism of nonrhizospheric soil, present much more accentuated antagonism when cultivated together on soil extract medium than on root extract. The inhibi-

tion of *Arthrobacter globiformis* is said to be due to acidification of the culture medium by *Pseudomonas* sp., and perhaps also to production of antibiotics, favored by their culture medium. The same investigators simultaneously inoculated a root extract medium with *Agrobacterium radiobacter, Arthrobacter citreus, Azotobacter, Bacillus cereus,* and *Pseudomonas* and found a clear-cut inhibition of *Bacillus,* a less marked inhibition of *Azotobacter chroococcum,* and, in contrast, a predominance of *Pseudomonas,* a result similar to the observed distribution in the natural rhizosphere (Chan *et al.,* 1963).

Antibiosis is a particularly spectacular form of antagonism, observed *in vivo,* but its importance in the rhizosphere ranks alongside others, as that stemming from the competition for nutrients or space and the antagonism between parasitic organisms. The latter types of antagonism result in roots being protected from pathogenic microorganisms. Competition among rhizospheric microorganisms suggests that in this environment the sources of nutrition and energy are limited. The frequency with which *Fusarium* is isolated in the rhizosphere is due to the great competitive power of this fungus.

Chalvignac (1966) noted that the selective effect of root exudates on the microflora is the result of competition which occurs among the slow- and rapid-growing species and that the latter are particularly favored in the rhizosphere.

Competition plays an important role in protecting plants attacked by pathogens. In this respect, Katznelson (1965) showed that rhizospheric and soil bacteria can limit the development of a pathogenic fungus of alfalfa roots by competing with the fungus for thiamine and mineral nutrients.

The microbial formation of antibiotics is often greater in the rhizosphere than in nonrhizosphere soil. These microorganisms are essentially actinomycetes whose antagonism is exerted toward bacteria (e.g., *Azotobacter* or *Rhizobium*) or toward fungi. In the rhizospheric environment, production of antibiotics by microorganisms can be shown quite easily, but it is more difficult to determine the actual role of these microorganisms. Production of antibiotics by rhizospheric microorganisms today cannot be denied, even if their presence sometimes seems exceptional. Strzelczyk (1966) found that resistance of certain varieties of flax (*Linum usitatissimum*) to *Fusarium oxysporum, F. lini,* and a number of varieties of tobacco to *Thielaviopsis basicola* is due to the presence in the rhizosphere of a large number of bacteria having great competitive power against fungi and not to the presence of bacteria which synthesize antibiotics.

6. Nutrition of Rhizospheric Microflora

The quantitative and qualitative differences between rhizospheric and nonrhizospheric microorganisms are due, above all, to their different sources of nutrition. Whereas the microorganisms which live in soil are not influenced by root systems because they feed on organic residues in varying stages of decomposition, the rhizospheric microorganisms receive their nutrients from root exudates. These substances, although not always readily utilized by microorganisms, are of great importance to the population of the plant's root surface and, under all circumstances, are the factors which determine the rhizospheric effect (Vančura, 1964).

The process of root exudation is still little understood, since plant physiologists have taken greater interest in root absorption than the reverse process. One of the most interesting methods for demonstrating root exudation consists of applying radioactive substances to the foliage and identifying the radioactive element at the root level. In this way Rakhtcenko (1958) found that after injecting ^{32}P in the leaves of forest trees this element was present in the soil at a distance of 0.25–2 m from the stem. Indirect proof of root exudation was observed in situations where the application of certain substances to leaves led to a spectacular change in the rhizospheric microflora.

According to Katznelson *et al.* (1955), alternating wet and dry conditions in the soil foster exudation of amino acids. High light intensity and temperatures also activate exudation (Vančura, 1967).

Studies on the nature of root exudates are numerous, but it is not possible to give any general rules about the qualitative composition of exudates. This varies with each variety (which clearly explains the great specificity of the rhizospheric effect) and with the state of development of the plant. Melin (1962) demonstrated the presence of a factor strongly stimulating development of mycorrhiza-forming mycelia in pine root culture.

Slankis *et al.* (1964) investigated the composition of root exudates of seedlings of white pine (*Pinus strobus*), grown for 9 months in a controlled environment. Using $^{14}CO_2$ they identified 35 components including two sugars (glucose and arabinose), two amides (glutamic acid and asparagine), and six organic acids (glycolic, malonic, malic, shikimic, oxalic, and *cis*-aconitic acid). Vančura (1964) studied the root exudates of barley and wheat plants in their initial growth stages, and showed through chromatographic separation the following sugars in wheat: maltose, glucose, arabinose, xylose, ribose, and rhamnose; and the following sugars in barley: maltose, galactose, glucose, arabinose, xylose, ribose, deoxyribose, and another unidentified sugar. Vančura

and Hovadik (1965), studying the composition of the root exudates found fourteen sugars in watermelon (*Cucumis sativus*),three sugars in turnip cabbage (*Brassica oleracea* v. *gongylodes*), eleven sugars in tomatoes (*Lycopersicon esculentum*), and nine sugars in red peppers (*Capsicum annuum*). Greater quantities of arabinose were found in watermelons, and greater quantities of fructose in tomatoes and red peppers. In turnip cabbage only two unidentified oligosaccharides and xylose were found. These sugars diminish in quantity as the various plants approach flowering stage. Rovira (1956), found that when green peas and oats were grown aseptically in sterile sand, fructose and glucose were exuded by the roots of both plants only during the first 10 days of growth.

Various writers have used the electron microscope to demonstrate a layer of mucilaginous substances or mucigel around the root tips of many plants. Such layers, which are several microns in thickness, apparently are composed of polymers of pectic substances (Jenny and Grossenbacher, 1963; Dart and Mercer, 1964; Rovira, 1965a,b). This mucigel is not only a source of carbohydrates for the rhizospheric microflora, but it also changes the rhizospheric environment, fixing enzymes of plant or microbial origin and extending the ion exchange area around roots.

Root exudates contain many amino acids, such as leucine, isoleucine, valine, glutamine, α-alanine, β-alanine, asparagine, serine, glutamic acid, aspartic acid, glycine, phenylalanine, threonine, tyrosine, proline, lysine, methionine, cystathionine, γ-aminobutyric acid, and tryptophan. The list of these compounds varies considerably according to growing conditions and the plant varieties considered. Scheffer *et al.* (1964) identified homoserine, threonine, α-alanine, glutamine, asparagine, and serine in root exudates of peas, and serine, lysine, and glycine in those of oats.

Quantitative data on production of amino acids are rare. However, an investigation by Paul and Schmidt (1961) is noteworthy because it showed that the rhizospheric soil of soybean contained from 2 to 4 μg of amino acids per gm of soil.

According to Bowen (1969), exudation is directly related to the nutritional state of the plants. He detected greater release of amides and amino acids in *Pinus radiata* seedlings that had developed in nutrient solution lacking phosphates than in plants in complete nutrient solutions. This was ten times greater in plants that had grown in a solution lacking nitrogen.

Vančura (1964) identified fourteen and eighteen amino acids, respectively, in root exudates of barley and wheat. The common amino acids were asparagine, aspartic acid, serine, glycine, glutamic acid, threonine, α-alanine, proline, tyrosine, phenylalanine, isoleucine, and leucine. Those unique to barley were cysteic acid and α-aminoadipic acid, and those

unique to wheat were cysteine, cystathionine, glutamine, β-alanine, and γ-aminobutyric acid. Vančura and Hovadik (1965) observed that in watermelon the chief amino acid was β-pyrazolylalanine, which was also present in the free state in the seeds. They observed that turnip cabbage exudates contained eighteen amino acids, with larger quantities of glycine, α-alanine, leucine, and methionine, and that tomato roots in their initial growth stage contained fifteen amino acids, with large quantities of glutamic acid, threonine, and α-alanine, while citrulline, ornithine, and proline were present only as traces. At the flowering stage, tomato root exudates contained tyrosine and three unidentified substances in addition to those found earlier. At this stage the quantities were generally smaller. This means that during the vegetative period of growth quantitative changes occur in the distribution of the individual components of root exudates. They also observed that the root exudates of red peppers contained eighteen amino acids in the initial growth stage, with larger quantities of serine, glycine, threonine, and glutamic acid, while in the flowering stage, tyrosine and six further unidentified substances were found with a relatively greater quantity of asparagine.

Rovira (1956), studying the root exudates of pea and oat plants grown aseptically in quartz sand, noted that the pea plants contained amino acids in larger proportions than in oat plants during the first 21 days of growth. In later studies Rovira (1959) found that the root exudates of tomato (*Lycopersicum esculentum*), subterranean clover (*Trifolium subterraneum*), and *Phalaris tuberosa*, produced in nutrient solutions under different environmental conditions, differed in the amount of amino acids exuded in the three types of plants. It was larger in *Phalaris tuberosa* than in tomato. Exudation appeared more abundant during the first 2 weeks of growth than in the following 2 weeks. Light intensity proved to have a strong influence on root exudation of amino acids in clover. In the exudates of tomato, he found a diminution of aspartic acid, glutamic acid, phenylalanine, and leucine as the light diminished, while serine and asparagine appeared to increase. Finally, high temperatures increased the quantities of amino acids exuded by the roots of tomato and subterranean clover. Furthermore, a number of vitamins were isolated from the root exudates; these included biotin, thiamin, calcium pantothenate, niacin, and riboflavin. Biotin is frequently found in sufficiently high concentrations to be biologically active.

Rovira subsequently (1969) reviewed the role of root exudates and provided interesting data about their nature, the factors which influence exudation, the mechanism of exudation, and the influence of exudates on terrestrial microorganisms.

Sulochana (1962), while examining the root exudates of diploid and

amphidiploid strains of cotton in soils with and without *Fusarium*, found choline, pyridoxine, *p*-aminobenzoic acid, and traces of biotin and inositol in the infected soil. In the noninfected soil he found pyridoxine, choline, thiamine, *p*-aminobenzoic acid, and traces of biotin and inositol. Thiamine, biotin, pyridoxine, and *p*-aminobenzoic acid were found in greater quantities in the root exudates of diploid cotton strains grown in soils that were not infected by *Fusarium*. The amphidiploid strains, with very few exceptions, show comparatively smaller quantities of vitamins in the root exudates. It is probable that the vitamin needs of terrestrial microorganisms are satisfied not only by production of root exudates, but also by other rhizospheric microorganisms with which they live in association, and that they are capable of synthesizing vitamins.

Organic acids have also been identified in root exudates. These include formic, acetic, propionic, butyric, valeric, glycolic, oxalic, succinic, fumaric, malic, tartaric, nitric, pyruvic, and oxalacetic. The acids can be exuded in relatively large quantities. Thus a single wheat plant can, up to tillering, viz., in 6 weeks, free 13 mg of acetic acid, 3.5 mg of propionic acid, 2 mg of butyric acid, and 1.5 mg of valeric acid (Rivière, 1959, 1960). Therefore, it is likely that the organic acids from the exudates play an important role in the rhizosphere, as energy substrates for microorganisms and as acidifying agents or in chelating metal ions. Vančura (1964) identified the following organic acids in root exudates of barley and wheat: two keto acids (pyruvic and oxalacetic) and a number of hydroxy acids and di- and tricarboxylic acids (oxalic, malic, glycolic, succinic, and fumaric).

Vančura and Hovadik (1965) identified lactic and oxalic acids in root exudates of watermelon. In the exudates of turnip cabbage they found, in addition to the above-mentioned acids, malic and glycolic acids, while the root exudates of tomato and red peppers, in the initial growth stage, have the same organic acids (oxalic, citric, malic, glycolic, succinic, and fumaric). These same organic acids were identified in root exudates of tomato during the flowering stage, but in distinctly smaller quantities. Oxalic, citric, and fumaric acids were identified in root exudates of red pepper during flowering, but the other organic acids produced during the initial stage of growth were not noted.

7. Other Factors Influencing Composition of the Rhizosphere

Rhizospheric soil is generally richer in enzymes than is nonrhizospheric soil. The enzymes are of both plant and microbial origin. Enzymes of plant origin may be exuded or arise from deteriorating root cells. The latter case, however, is not one of exudation in the strict sense. Consid-

erable work has been carried out on root exudation of enzymes. Knudson (1920) found glucose and fructose present in a saccharose solution in which plants developed aseptically. He claimed that this indicated that the root exudes invertase. Subsequently, however, Knudson (1920) showed that the saccharose is first absorbed by the roots and then hydrolyzed to glucose and fructose, and small amounts of these sugars are then exuded. Krasilnikov (1952) identified invertase, amylase, and protease enzymes in wheat, maize, and pea plants. The enzyme polygalacturonase was identified in exudates of alfalfa and clover. This enzyme manifests itself only in the presence of *Rhizobium* sp. Krasilnikov and Kotelev (1959) established that the enzyme phosphatase is exuded by maize. When the atmosphere is saturated with moisture, plants are able to exude appreciable amounts of water (Schippers *et al.*, 1967).

Apart from the substances listed, products derived from degradation of nucleic acids, flavones, glucosides, etc., have recently been discovered in root exudates. The exudates are not released uniformly along the whole root system, instead they are produced in greatest concentrations in the root elongation zone and at the root hairs, i.e., where metabolic activity is more intense and permeability is greatest.

Quantitative data on the amount of root exudates are relatively rare and, except for some exceptions (Harmsen and Jager, 1963), they have been established on the basis of hydroponic cultures which certainly do not reflect the course of the phenomenon in nature.

According to Demidenko (quoted by Macura, 1966), the weight of the root exudates accounts for 27% of the plant mass (wheat and tobacco). Vančura (1961), however, proposed considerably lower values, e.g., 7–10%. These assessments are almost of the same order or slightly higher than Meschkov's (quoted by Macura, 1966); according to the latter investigator, the exudates of maize, expressed as carbon, represent 1.0–1.7% of the plant mass. It is therefore probable that production of exudates in soil is at least as great as in hydroponic cultures, since they have undergone biodegradation by the rhizospheric microflora or have been absorbed by soil colloids.

While research on chemical composition of root exudates has increased rapidly in the past 10 years, information on the effect of these exudates on the different sectors of the pathogenic or saprophytic microflora is still incomplete. One of the major difficulties is the fact that the action of the exuded substances varies according to their concentration in the rhizosphere (Daste, 1956) and that the concentration of these substances, their area of diffusion, their importance, and the speed of their inactivation through biodegradation or through absorption of soil colloids are not known. The variations in concentration of

biologically active substances in root exudates could explain the sequence of the phenomena of stimulation and depression frequently observed in certain microorganisms, such as *Azotobacter* sp. and nitrite-forming bacteria (Molina and Rovira, 1964). However, there are cases in which the stimulating or inhibiting effect is well established in particular experimental conditions. These quantitative and qualitative indications are also incomplete because in the majority of studies the fraction or fractions derived from metabolism of the symbiont fungi, normally found on the root tips in symbiotic association, are not considered. Above all, in plants characterized by ectotrophic mycorrhizal symbiosis, the contribution of these metabolites is undeniable and significant enough to modify the composition of the exudates which are liberated in the adjacent soil.

According to Rubenchik (1960), *Beijerinckia indica* is stimulated by root exudates of sugar cane (*Saccharum officinarum*), and *Azotobacter chroococcum* is stimulated by exudates of *Canna indica*. Cátská (1965) ascertained that fungi (*Rhizopus arrhizus*, *Mucor racemosus*, *Fusarium oxysporum*, and *Penicillium chermesinum*) isolated from the root surface of wheat fructify better when they are close to germinating plants than in the soil.

Experiments with pathogenic fungi (*Fusarium oxysporum*, *F. pisi*, and *Plasmodiophora brassicae*) show that plants resistant to these parasites exude substances which inhibit spore production, whereas susceptible plants stimulate this process (Buxton, 1957; Bochow, 1965). However, the stimulatory effect of root exudates on spore production in pathogenic fungi does not always manifest itself. Schroth and Hendrix (1962) observed that formation of chlamydospores in *Fusarium solani f. phaseoli* took place in the presence of exudates of susceptible and resistant plants.

According to Samtsevich (1965), during the first stages of growth, roots produce considerable quantities of toxic substances, limiting microbial development; this later diminishes, thereby permitting formation of the rhizospheric zone by the soil microorganisms. Certain toxic substances have been isolated and identified. In the root tips of oats, Schönbeck (1958) isolated a toxic glucoside which inhibited certain fungi. Various phenolic substances (isochlorogenic acid, chlorogenic acid, gallotannic acid, and gallic acid) have also been isolated (Vančura, 1964; Rice, 1967). These inhibiting substances play a dual role. First they protect plants against infection by pathogenic organisms such as fungi, bacteria, and possibly viruses. As shown earlier, plants that are resistant to pathogenic fungi owe this property to the nature of their exudates which inhibit the germination of spores of the parasite. This

situation received further confirmation from Strzelczyk's (1961) work on flax. The varieties resistant to *Fusarium* were those which exuded substances limiting the development of the rhizospheric micropopulation. Second, competition among plants is seriously affected. Rice (1967) showed that a number of plants requiring little mineral nitrogen (*Ambrosia elatior, Euphorbia corollata,* and *Helianthus annuus*) produced inhibitors of nitrifying and nitrogen-fixing microorganisms that gave them a considerable advantage over plants whose nitrogen needs were greater. This competitive mechanism would appear to slow down the speed of succession of plant species.

8. *Conclusions*

From all these investigations it is clear that formation of the rhizospheric environment proceeds in ways and with mechanisms that are substantially common to the majority of plants. However, the phenomenon is not unexpectedly linked also to multiple environmental factors and to certain individual characteristics of the plant.

There is little doubt that with regard to the formation of the rhizospheric environment, a broad division could be made between mycorrhizal and nonmycorrhizal plants. The former, which are more common, certainly produce stimulatory effects in the soil microbial environment that are substantially different from the latter. The latter condition is encountered less frequently but must necessarily be taken into consideration, since several investigations have been conducted on plants grown in unnatural conditions that do not permit mycorrhizae to form.

Mycorrhizal roots could be divided further into ecto- and endomycorrhizae. The former include nearly all forest plants, and the rhizospheric phenomenon is typical of plants characterized by a metabolism linked to slow regular growth in equilibrium with the environment. These conditions affect a large volume of soil, and the mycorrhiza-forming fungus plays a very important role. The second group is characteristic of most herbaceous plants, and the rhizospheric phenomenon is typical of fast-growing plants that have a relatively intense metabolism in a moderate soil volume in which the mycorrhiza-forming fungus plays a lesser role.

There is little doubt that metabolism of these plants is profoundly influenced by the presence of mycorrhizae. Hence studies on the formation and composition of the rhizosphere should be made on mycorrhizal plants. Many workers, as far as can be ascertained, have transferred results obtained from laboratory studies on nonmycorrhizal plants to the natural environment, without considering that, under natural conditions, they may form mycorrhizae and as a consequence their rhizosphere environment would be completely different.

The trees forming the forests in tropical and equatorial areas must be considered separately. In these areas, the majority of the trees are characterized by having endomycorrhizae; their root systems occupy a large volume of soil and very often develop with great rapidity and thus possess a very active metabolism. The formation of the rhizospheric environment in these plants, the type of stimulation, and the interactions that develop in these ecosystems have been little studied and deserve greater attention.

B. Formation of the Rhizosphere Ambient

The rhizosphere is characterized by a particular type of microbial population (bacteria, actinomycetes, and fungi) that is unique in its quantitative and qualitative features. In order to understand the formation of this environment, a hypothetical soil will be considered that is absolutely devoid of vegetation in which the microorganisms are evenly distributed and live together in perfect ecological equilibrium. The germination of the seed of a higher plant in such a soil, and the penetration and development of a rootlet, will cause natural and obvious changes in the balance of the microbial population. These changes stem not only from the absorption of nutrient substances by the growing plant, which are taken away from the terrestrial microflora, but also from secretion of the by-products of the plant's metabolism in the soil around the root tips. These substances, or "root exudates" have, as indicated, a complex composition. They contain organic acids, vitamins, free amino acids, and large protein molecules.

The accumulation of exudates near the root tips stimulates multiplication of all the microbial forms (bacteria, actinomycetes, and fungi) present in the soil around the tips, which are able to break the protein macromolecules and to use them, at least in part, for nutritional purposes. The multiplication of these microbial forms inevitably inhibits the development of others which are unable to break down the protein macromolecules and are thus at a disadvantage in the particular nutritional environment created.

Fragments of the protein macromolecules are released after they have been partially utilized by the microbes. These substances are displaced by new exudates and by solutions circulating in the soil, and in this way they come into contact with other terrestrial microorganisms which further degrade them for nutritional purposes, thereby reducing them to still simpler forms. The whole system of rhizospheric microorganisms therefore undergoes change, starting from the root surface and gradually moving through the soil away from it, for a distance of up to a few millimeters. If these microorganisms have the capacity of using the

substances normally secreted by the root tips, consideration must also be given to their distribution because of their intimately interlinked metabolic capacities.

It is easy to understand from the above how well-balanced microflora in the hypothetical soil evolved toward another form of equilibrium, regulated and conditioned by factors outside of the microbial ambient, i.e., by vegetational factors, typical of the majority of ecological relationships.

Naturally, the rhizospheric microbial groups will become more and more defined by the development of plants and, consequently, by the quantity of catabolites produced by their roots. In view of the composition of the root exudates, the ammonifying group of bacteria is normally well represented in the rhizosphere. These microorganisms are able to attack proteins, and, as the final stage in the degradation process, produce ammonia.

Fungi are also abundantly represented in the rhizosphere. Recent investigations, however, have brought to light a certain specificity between the rhizospheric fungal population and the host. In this way, each host, on the basis of the constituents of the root exudates produced, seems able to select a given fungal microflora, and among them a number of species are dominant.

C. Formation of Mycorrhizae inside the Rhizospheric Ambient[2]

The mycorrhiza-forming fungi are undoubtedly among those best able to degrade the protein molecules which comprise the root exudates. For adequate understanding of formation of mycorrhizae in the rhizosphere, the pedological ambient described previously will again be considered. Seed germination, development of vegetation, and liberation of root exudates in the zone of soil surrounding the tips gave rise, as seen, to ordered development of given microbial groups. Among the complex of terrestrial microflora there will also be mycelial fragments, and spores of mycorrhiza-forming fungi. Germination of these propagules results in production of a mycelial thread which will spread out in the surrounding soil. When the root exudates, even if considerably altered by rhizospheric microflora, come into contact with mycelium of the mycorrhiza-forming fungi, the hyphae are stimulated to develop, especially in the direction of the zones where such exudates are in the greatest concentrations. The mycorrhiza-forming hyphae thus compete with the rhizospheric microflora for these exudates and develop toward the source of these substances until the root tip is reached. Once the tip has been

[2] See Chapters 1, 4, 6, and 7.

reached, they colonize the root surface and utilize the exudates in active competition with the other microorganisms.

At this point, the fungus covers the root tips with an initially loose mycelial network, which becomes increasingly dense until it forms the "mycoclene" or fungus cover. The fungus then penetrates the root tissues of the host and starts the process of mycorrhizal symbiosis.

From this moment on the rhizosphere phenomena undergo a total change. The exudates first liberated directly in the soil around the tips are now filtered by the fungus cover and are partially utilized by the mycorrhizal fungus for nutritional purposes. The exudates are modified profoundly when they reach the soil, especially by products of metabolism of the mycorrhiza-forming fungus. In the soil around the mycorrhizal root tips very complex substances are now released which can be attacked and utilized, at least partially, only by certain microorganisms capable of metabolizing them. Parallel to these developments in the rhizospheric ambient, the first metabolites released are degraded into simpler forms that could be used by microorganisms with less demanding nutritional requirements. In this way a distribution pattern of the rhizospheric microorganisms can be imagined, beginning from the mycorrhizae, with which they are intimately connected, and extending into the surrounding soil. These microorganisms are thus in a balanced and interrelated distribution, depending on their ability to utilize root and mycorrhizal exudates.

D. Concept of the Mycorrhizosphere

Root exudates and metabolites of the mycorrhiza-forming fungus are therefore the basis of microbial stimulation which precede formation of the rhizospheric ambient of the mycorrhizae. Not many studies exist on this subject. Tribunskaya (1955), comparing the rhizosphere of mycorrhizal and nonmycorrhizal pine seedlings, identified a considerable increase and a change in composition of the rhizospheric microflora around the mycorrhizal rootlets. The author found nine to ten times as many fungi in the rhizosphere of the latter and a large number of bacterial saprophytes and bacteria capable of degrading organic substances. In both rhizospheric ambients investigated, the development of *Azotobacter* was inhibited in some way. Ivarson and Katznelson (1960) studied the rhizosphere of birch seedlings grown in forest soil under controlled conditions. They probably used mycorrhizal plants. A marked rhizospheric effect was noted, especially 28 weeks after the end of the dormancy period of the seedlings. This effect was associated with an increase in the number of ammonifying bacteria, that reduced methylene blue, and with

gas-producing anaerobes. Later, Katznelson *et al.* (1962) studied the rhizosphere in mycorrhizal and nonmycorrhizal *Betula alleghaniensis* roots. In this case, also, the rhizospheric effect was greater in the mycorrhizal rootlets, and they identified a large number of bacteria and actinomycetes, as well as bacteria capable of reducing methylene blue, fermenting glucose, and promoting ammonification.

Oswald and Ferchau (1963) carried out investigations on aerobic bacteria associated with mycorrhizal and nonmycorrhizal rootlets of *Picea engelmannii, P. pungens, Pinus aristata, P. flexilis,* and *Pseudotsuga menziesii.* They identified 51 species of bacteria in 253 isolates, including 22 associated with mycorrhizae, 7 with nonmycorrhizal rootlets, and 22 common to both types of rootlets. Neal *et al.* (1964) examined the rhizospheric microflora associated with mycorrhizae of *Pseudotsuga menziesii* and found stimulation of the bacteria in various mycorrhizal forms, but compared with nonmycorrhizal and suberized rootlets, no great difference appeared, in favor of the former, regarding the morphological and physiological forms of the isolates.

Foster and Marks (1967) examined the mycorrhizospheres of *Pinus radiata* with an electron microscope, and found various morphological types of bacteria associated with different types of mycorrhizae. Rambelli (1970) identified, as noted previously, a quantitatively different population of oligonitrophilous bacteria in mycorrhizal and nonmycorrhizal rootlets of *P. radiata.* These bacteria possibly belong to a single species, and are present inside the fungus cover in mycorrhizae produced by *Boletus granulatus.* They appear capable of fixing small quantities of atmospheric nitrogen.

One of the most interesting and little understood aspects of the formation of the rhizospheric ambient of mycorrhizae, especially in cases of ectendotrophy, is the complex mechanism of antibiotic production[3] by the mycorrhiza-forming fungi. The production of antibiotics by basidiomycetes has been known for some time, and the bibliography contains long lists of species capable of liberating these complex substances in culture substrates (Wilkins and Harris, 1943a; Wilkins, 1945, 1947; Mathieson, 1946; Robbins *et al.*, 1946; Santoro and Casida, 1962; Krywolap and Casida, 1964; Sásek and Musilek, 1967, 1968; Musilek *et al.*, 1969). However, the phenomenon in nature is less known, especially when the mycorrhiza-forming fungi are associated with the root tips to form the mycorrhizae. Krywolap *et al.* (1964) discovered an antibiotic substance in extractions made with acetone or methanol from mycor-

[3] See Chapter 9.

rhizae formed between *Cenococcum graniforme* and the roots of white pine (*Pinus strobus*), red pine (*P. resinosa*), and Norway spruce (*Picea abies*). The authors also found these substances in the root tissues and needles of these plants, and suggested that they migrated from the mycorrhizal fungus.

Nothing, however, is known of the possible liberation of antibiotic substances by mycorrhizae in the soil around the mycorrhizal tips. This seems plausible even though, as far as is known, it has never been proven. The formation of the rhizospheric ambient around the mycorrhizal tips would, in this case, depend on two contrasting mechanisms (i.e., microbial stimulation, due to the production of root exudates liberated by the mycorrhizal fungus, and microbial inhibition, due to production of antibiotics) which would inhibit sensitive microorganisms. This dual mechanism would result in true microbial selection and formation of an undoubtedly special rhizospheric ambient.

Even if these observations are hypothetical for the moment, the mycorrhizospheric ambient seems clearly delimitable. Undoubtedly the fungal symbiont intervenes, modifying the previously formed rhizospheric ambient, changing the composition of the substances which are liberated into the soil surrounding the root tips. Even if we consider that mycorrhizae originated within the rhizosphere, through the interaction of microbial stimulation by the root exudates and by selection by microbial competition, the mycorrhizal fungus will dominate the other microorganisms and colonize the root tips. The changes wrought by this fungus on the residual rhizospheric microflora are profound and permanent. In the author's view it appears justifiable to apply the name "mycorrhizosphere" to this particular microbial ambient. Perhaps the distinction between the mycorrhizosphere and rhizosphere might also apply to the microbial ambient around the mycorrhizae. The latter will include all those microorganisms located further from the surface of the fungus cover and influenced either very slightly or not at all by the action of the substances produced by the symbiont. However, they are still stimulated to multiply by residues of substances partly utilized by the mycorrhizospheric microflora.

II. Distribution of Rhizospheric and Mycorrhizospheric Microorganisms over the Four Seasons

Reference has already been made to the work of many investigators on the qualitative distribution of rhizospheric microorganisms in various

plants (Tribunskaya, 1955; Ivarson and Katznelson, 1960; Katznelson *et al.*, 1962; Neal *et al.*, 1964; Oswald and Ferchau, 1963). Skyring and Quadling (1969) developed noteworthy techniques, and they were able to characterize the rhizospheric ambient by subjecting 400 bacterial strains isolated from the rhizosphere to 98 tests. In this way they identified characteristics typical of the rhizospheric microorganisms which proved completely different from those of the bacteria in the control. De Leval and Remacle (1969) followed changes in the rhizospheric microflora of *Populus canadensis* v. *robusta* at different periods of development. Using the techniques of Pochon *et al.* (1954), they examined the total microflora, aerobic and anaerobic nitrogen fixation, proteolysis, ammonification, denitrification, amylolysis, pectinolysis, hemicellulolysis, aerobic and anaerobic cellulolysis, and other physiological properties. Rambelli *et al.* (1972), in a recent investigation of the mycorrhizosphere of *Pinus radiata*, studied the variation of the rhizospheric microbial population for the whole range of seasons. Applying the techniques of Pochon *et al.* (1954) and those of Lochhead and Chase (1943), they studied the fungal, bacterial, and actinomycetic populations by making isolations from the soil immediately around the mycorrhizal root tips (first isolation) as well as isolations from the surface and within the mycorrhizae (second isolation). A brief review of some of these results follows (Rambelli *et al.*, 1972).

A. The Fungi

1. Identification

Rambelli *et al.* (1972) noted an extremely irregular distribution of the microflora throughout the four seasons. Isolations made from the adhering rhizosphere (second isolation) produced negative results in spring and winter. The first isolation, made in spring, produced only a few fungi representing few species. In summer, however, there was a considerable group of *Penicillia*, dominated mainly by *Penicillium corylophilum*, *P. asperum*, and another unidentified species. *Penicillium corylophilum* was present in almost all the isolations made during the four seasons, it proved dominant in autumn (first and second isolation) and, together with *P. waksmanii*, *P. vinaceum*, and an undetermined species of *Penicillium*, it was dominant in the winter (first isolation) (Table V).

These results do not agree with those obtained by De Leval and Remacle (1969), Katznelson *et al.* (1962), or Tribunskaya (1955). However, it is often difficult to compare results obtained by different authors, since identification of the fungal population isolated is not always complete.

TABLE V

FUNGI ISOLATED FROM *Pinus radiata* RHIZOSPHERE IN THE FOUR SEASONS

Species	Spring		Summer		Autumn		Winter	
	I	II	I	II	I	II	I	II
Penicillium thomii	1	—	—	—	—	—	—	—
Penicillium cyclopium	—	—	2	1	3	2	1	—
Pen. cyclopium v. *echinulatum*	—	—	1	—	—	—	—	—
Penicillium frequentans	—	—	2	—	—	—	—	—
Penicillium corylophilum	1	—	18	1	8	4	23	—
Penicillium vinaceum	—	—	1	—	—	—	6	—
Penicillium raistrickii	—	—	1	—	—	—	—	—
Penicillium sclerotiorum	—	—	1	—	—	—	—	—
Penicillium janthinellum	—	—	—	—	1	—	—	—
Penicillium restrictum	—	—	—	—	1	—	1	—
Penicillium oxalicum	—	—	—	—	—	1	—	—
Penicillium lilacinum	—	—	—	—	—	—	1	—
Penicillium waksmanii	—	—	—	—	—	—	14	—
Penicillium asperum	—	—	4	—	—	—	—	—
Penicillium sp. No. 1	1	—	—	—	—	—	—	—
Penicillium sp. No. 2	1	—	—	—	—	—	—	—
Penicillium sp. No. 3	—	—	10	—	—	—	—	—
Penicillium sp. No. 4	—	—	—	—	—	1	8	—
Aspergillus fumigatus	—	—	—	—	—	—	1	—
Aspergillus versicolor	—	—	1	—	—	—	—	—
Spicaria sp. No. 1	—	—	—	—	6	—	—	—
Spicaria violacea	—	—	3	—	2	—	3	—
Beauveria bassiana	1	—	—	—	—	—	—	—
Cladosporium sp. No. 1	1	—	1	1	—	—	—	—
Cephalosporium sp. No. 1	1	—	1	—	1	—	—	—
Phoma sp. No. 1	1	—	—	—	—	—	—	—
Chaetomium spinosum	—	—	1	—	—	—	—	—
Verticillium sp. No. 1	—	—	—	—	1	—	—	—
Mortierella alpina	—	—	—	—	1	—	—	—
Rhizopus sp. No. 1	1	—	—	—	—	—	—	—
Micelia sterilia	2	—	4	—	5	—	2	—

B. THE BACTERIA

1. Nitrogen Fixation

Investigations on seasonal nitrogen fixation in the mycorrhizosphere are relatively scarce. Most of these have been conducted on the rhizosphere of legumes, probably in an attempt to identify rhizospheric stimulation by root symbiont bacteria. Rangaswami and Vasantharajan (1962a,b), who studied the rhizosphere of citrus, identified an appreci-

TABLE VI

DISTRIBUTION OF RHIZOSPHERIC MICROORGANISMS WITH DIFFERENT PHYSIOLOGICAL CHARACTERS IN THE FOUR SEASONS. DATA EXPRESSED IN PERCENTAGE OF TOTAL NUMBER OF BACTERIA TESTED

	N fixation		Ammonification		Nitrite formation		Nitrate formation		Denitrifaction		Not identified	
	I	II	I	II	I	II	I	II	I	II	I	II
Spring	18	25	69	79	0	0	0	0	9	0	4	0
Summer	5	17	38	67	0	0	0	0	4	0	53	16
Autumn	6	15	38	76	0	0	0	0	0	7	56	2
Winter	7	6	90	78	0	0	0	0	3	11	0	5

able population of nitrogen fixers, with R/S[4] indices ranging from 0.78 to 1.71 in the varieties considered. According to Tribunskaya (1955), the development of nitrogen fixers does not occur normally in the mycorrhizosphere and the rhizosphere of pine, which does not favor their multiplication. Maliszewska and Moreau (1959), who investigated the rhizosphere of *Abies alba*, did not identify any nitrogen fixers except *Clostridium* sp.

The investigations conducted by Rambelli (1970) agree well with those of Tribunskaya. Indeed, in the mycorrhizosphere of *Pinus radiata*, Rambelli found a certain percentage of microorganisms that grew well in a liquid mineral medium devoid of nitrogen. However, although he considered that these microorganisms were able to fix small amounts of atmospheric nitrogen, they were not true nitrogen fixers. In any case, the physiological characters of these bacteria are still extremely uncertain. Their quantitative distribution in the soil adhering immediately to the mycorrhizae over the four seasons is about 18% in spring, 5% in summer, 6% in autumn, and 7% in winter (Table VI). In isolations made from the actual mycorrhizal mantle, 25, 17, 15, and 6% were obtained during the four seasons. These microorganisms therefore seem to be located mainly on the surface of the fungus cover, and they probably receive positive stimulation from the fungal metabolites and root exudates.

By making isolations inside the mycorrhizae, and after sterilizing the outer surface of the fungus cover, Rambelli (1970) obtained, in a pure culture, the same microorganisms in the form of an extremely homogeneous microbial population, which showed good growth in a liquid mineral medium devoid of nitrogen as the only physiological characteristic.

2. *Ammonification*

This subject has received the most attention in the mycorrhizosphere (Tribunskaya, 1955; Ivarson and Katznelson, 1960; Katznelson *et al.*, 1962; Neal *et al.*, 1964; Oswald and Ferchau, 1963; Rambelli, 1966; Rambelli *et al.*, 1972). Over the four seasons, Rambelli identified the following variation in the quantitative distribution of ammonifying bacteria in the micorrhizosphere of *Pinus radiata:* spring, 69%; summer, 38%; autumn, 38%; and winter, 90% in the soil immediately adjoining the mycorrhizae, and on the surface of the fungus cover the variations were 79, 67, 76, and 78%. This group, therefore, also seems to be stimulated appreciably by fungal metabolites and root exudates. But their development, as noted earlier, should cause no surprise, in view of the enrichment of the ambient by easily metabolized sources of nitrogen through the seasons.

[4] R/S, rhizosphere–control soil.

3. *Nitrate Formation*

The interpretation of seasonal variations in nitrate formation is still open to considerable controversy. Under natural conditions the mycorrhizospheric ambient would not appear able to support any active nitrite- and nitrate-forming microflora. Remacle (1963), studying the rhizospheric effect in plants that colonize pedologically different environments, found in a number of cases a rhizospheric effect that stimulated the nitrite- and nitrate-forming microflora. In the rhizosphere of mycorrhizal plants of *Abies alba*, Maliszewska and Moreau (1959), however, did not find any nitrate-forming microorganisms. In preliminary investigations on the distribution of physiological groups in the mycorrhizosphere of *Pinus radiata*, Rambelli (1970) found a small percentage of nitrite- and nitrate-forming bacteria. In his subsequent study on the distribution of the mycorrhizospheric flora of *P. radiata* over the four seasons, the author did not find any nitrite- and nitrate-forming microorganisms (Table VI). Probably the techniques used for the study of this group of microorganisms in the rhizosphere are not the most suitable and should be appropriately modified in order to obtain more reliable results.

4. *Denitrification*

Physiological aspects of denitrification have not been fully studied in the rhizosphere (Ivarson and Katznelson, 1960; Katznelson *et al.*, 1962; Neal *et al.*, 1964; De Leval and Remacle, 1969; Rambelli, 1970). Rambelli *et al.* (1972) reported seasonal variation in distribution of denitrifying microorganisms in the soil surrounding the mycorrhizosphere of *P. radiata:* 9% in spring, 4% in summer, 0% in autumn, and 3% in winter. On the other hand, the following percentages of denitrifying microorganisms were isolated from the mycorrhizal mantle: 0% in spring and summer, 7% in autumn, and 1% in winter (Table VI).

5. *Proteolysis*

Proteolytic capacity of the rhizospheric bacterial flora has been studied by several investigators (Chalvignac, 1958; Rangaswami and Vasantharajan, 1962a,b; Remacle, 1963; De Leval and Remacle, 1969; Skyring and Quadling, 1969). However, since it is impossible to reproduce proteolytic capacity under conditions similar to nature, any practical study is nearly useless. Indeed, testing development of individual strains, isolated from the mycorrhizosphere, in the presence of fungal metabolites and root exudates, would have dubious value, because of their proteolytic action, sensitivity to growth inhibitors in fungal metabolites, and above all the difficulty of identifying the proportions of the two substances to

be tested. In any case, when we consider that the protein substances of the fungal metabolites and root exudates are mainly composed of amino acids, preliminary data can be obtained by examining ammonification of the individual rhizospheric strains isolated.

C. The Actinomycetes

Proteolysis

It is known from the literature that the actinomycetes as a group are stimulated to various degrees by root exudates and fungal metabolites. Maliszewska and Moreau (1959) noted a smaller number of actinomycetes in the rhizosphere of *Abies alba* than in control soil. Neal *et al.* (1964) identified a larger actinomycete population in suberized rootlets than in the mycorrhizae of Douglas fir. The opposite situation, however, is reported by many (Tribunskaya, 1955; Ivarson and Katznelson, 1960; Katznelson *et al.*, 1962; Rangaswami and Vasantharajan, 1962b; Rambelli, 1962, 1963, 1965, 1966). Thus, the behavior of the actinomycetes inside the mycorrhizosphere is still obscure. Indeed, considerable interest is attached to their presence in the soil near the mycorrhizae in numbers often far higher than that found on the fungal cover (Rambelli, 1962, 1963).

Because of their peculiar location, Rambelli carried out investigations on the behavior of actinomycetes isolated from the mycorrhizosphere of *Pinus radiata* in the presence of the metabolites produced by *Boletus granulatus*. The results, obtained during the four seasons of the year (Rambelli *et al.*, 1972), indicate, despite the limitations noted already, an almost total incapacity to attack the symbiont's protein substances. It is thus difficult to specify the role played by these actinomycetes inside the rhizosphere and the mycorrhizosphere. In any event, the subject deserves further investigation.

III. Relationships between Mycorrhizae and Rhizosphere

Root Exudates and Symbiont Metabolites

1. Influence of Root Exudates and Symbiont Metabolites on the Rhizosphere Microflora

It has already been shown that root exudates and fungal metabolites form the basis of the stimulatory processes that lead to formation of the mycorrhizospheric ambient. They provide a selective substrate for the terrestrial microflora, favoring development of certain forms and inhibit-

TABLE VII

TOTAL MICROFLORA GROWN WITH VARIOUS PERCENTAGES OF INTEGRAL OR BOILED CULTURAL SOLUTION OF *Boletus granulatus* AND ADDED TO SOIL AGAR

Percentages of culture solution	Raw cultural solution	Boiled cultural solution
Control	122,395,000	122,395,000
1	7,510,000	4,157,000
5	4,102,000	3,497,000
10	3,055,000	2,837,500
20	1,100,000	685,000

ing others; probably as indicated earlier, partly, through the effect of antibiotic substances produced by mycorrhizal fungi. But these substances not only inhibit given microbial groups, but also permit the indiscriminate development of others, so that, in a number of instances, there is an increase in the microbial flora in soils treated with antibiotics (Hervey, 1955).

Voznyakovskaya and Ryzhkova (1955) state, "There are certain facts

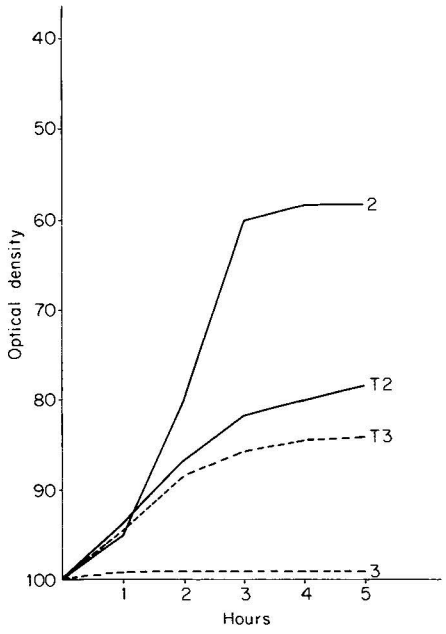

FIG. 1. Growth according to biophotometer of rhizosphere bacteria (strains 2 and 3) in a Lochhead mineral medium and root exudates (50%) of *Pinus radiata*.

indicating the dependence of mycorrhizal formation upon the presence of certain microbial associations." They consider that the prevalence of certain microbial groups in the mycorrhizosphere facilitates penetration of the symbiont into the host's root tissues. They reached this conclusion by simultaneously inoculating mycorrhiza-forming fungi and microorganisms isolated from the rhizosphere in seedlings grown in sterile soil. The attempt produced negative results. However, by adding 1 gm of forest soil to the pots containing sterile soil, mycorrhizal formation was able to proceed regularly.

Rambelli (1970) obtained interesting preliminary data on the influence of fungal metabolites on formation of the rhizospheric ambient. He tested the influence of culture solutions of *Boletus granulatus* on the microbial population of some good garden soil (Table VII). Thus, in the culture solutions of 60-day-old colonies of *B. granulatus* there are substances that inhibit microbial development. However, this is not indiscriminate but seems to take place through some type of selection process, since even 20% solutions do not inhibit many microorganisms. Consequently they could develop in the mycorrhizospheric ambient, favored also by the

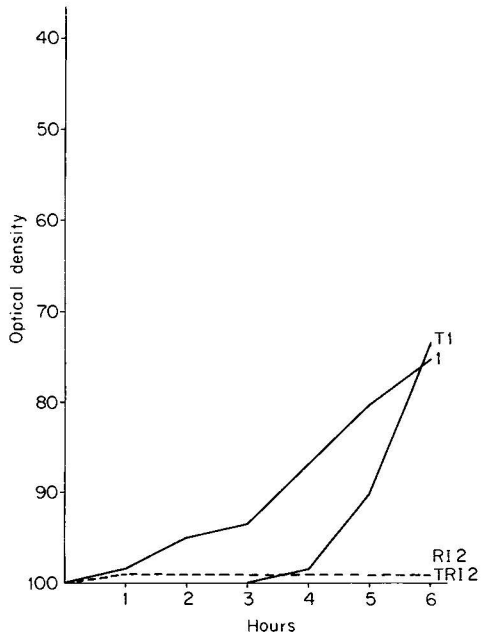

FIG. 2. Growth according to biophotometer of rhizosphere and mycorrhizosphere bacteria (strains 1 and RI2) in a Lochhead mineral medium and root exudates (50%) of *Pinus radiata*.

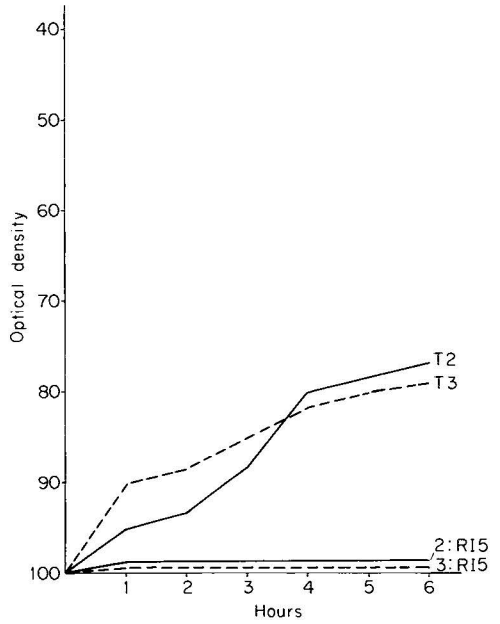

FIG. 3. Growth according to biophotometer of rhizosphere and mycorrhizosphere bacteria (strains 2, 3, and RI5) in a Lochhead mineral medium and root exudates (50%) of *Pinus radiata*.

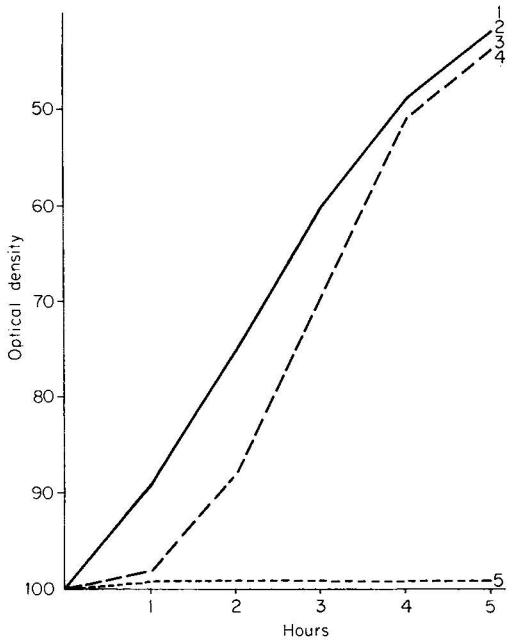

FIG. 4. Growth according to biophotometer of RI2 bacterial cells with 75% (No. 1), 50% (No. 2) and 25% (No. 3) metabolite produced by *Boletus granulatus*. Number 4 refers to the control and No. 5 to the integral metabolite.

lack of competition normally exerted by other terrestrial forms of bacteria. These results have suggested individual studies of the behavior of certain rhizospheric bacteria of *P. radiata* in the presence of root exudates and metabolites produced by *B. granulatus* (Figs. 1 and 2).

The investigations were conducted, above all, to check the influence of these substances on strain (RI2) isolated from inside the fungal mantle produced by *Boletus granulatus*, on root tips of *Pinus radiata*. Growth was measured with a biophotometer, and respiration was measured with the Warburg apparatus (Fig. 3). The root exudates generally stimulated respiration of certain rhizospheric strains and depressed others (Figs. 1–3). Regarding RI2, no stimulating action by root exudates was noted. The same strain, appeared to be stimulated considerably by large quantities of metabolites produced by *B. granulatus* (Figs. 4 and 5). These results seem to indicate that certain basic events affect the ecology of the bacterium RI2. This is a microorganism which is gram-positive, and in nitrogen-fixing agar forms round, bright, colorless,

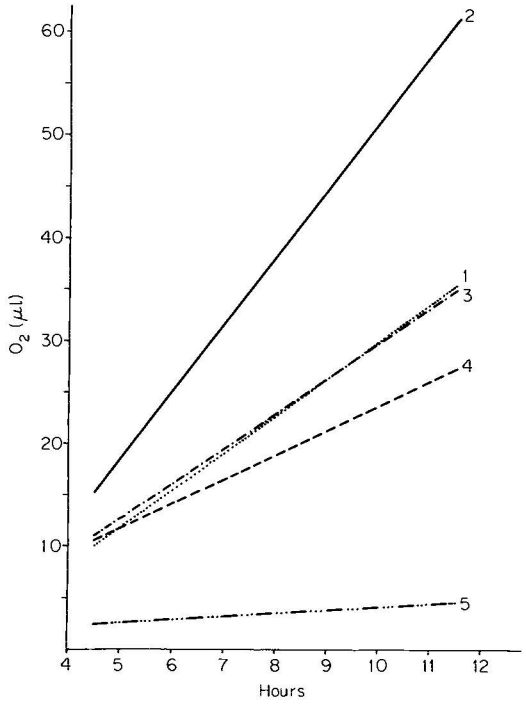

FIG. 5. Warburg respiration of RI2 bacterial cells with 75% (No. 1), 50% (No. 2), and 25% (No. 3) metabolite produced by *Boletus granulatus*. Number 4 refers to the control and No. 5 to the original metabolite.

highly mucous colonies. Under the microscope it appears in the form of thin rodlike cells, sometimes filamentous, or coupled, with one or two refractive polar drops. Rambelli studied its nutrition by cultivating it in various selective substrates. It grew well in a nitrogen-fixing substratum, and this would explain its inability to break down amino acids, cause nitrite and nitrate formation, and induce denitrification. Clearly, it utilized metabolites of the symbiont since it did not appear capable of using root exudates and the atmosphere as its source of nitrogen. It is possible that it was located inside the fungal mantle because of its particular requirements for nutrients or fungal hormones (Figs. 6 and 7).

Production of cytokinin has now been shown to occur in a number of mycorrhiza-forming fungi (Miller, 1967), and the presence of substances similar to kinetin in the root exudates of certain plants has been studied (Kende, 1965). It is not yet known what action these compounds exert on the rhizospheric microflora, nor, for that matter, is anything apparently known about diffusion of these substances in the soil around mycorrhizal

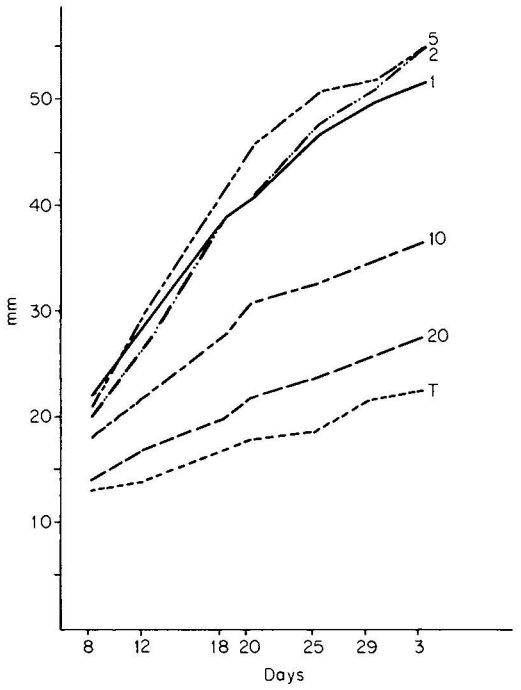

FIG. 6. Diameter increment of *Boletus granulatus* colonies in Hagem agar without malt, with varying percentages of metabolite obtained from strain RI2.

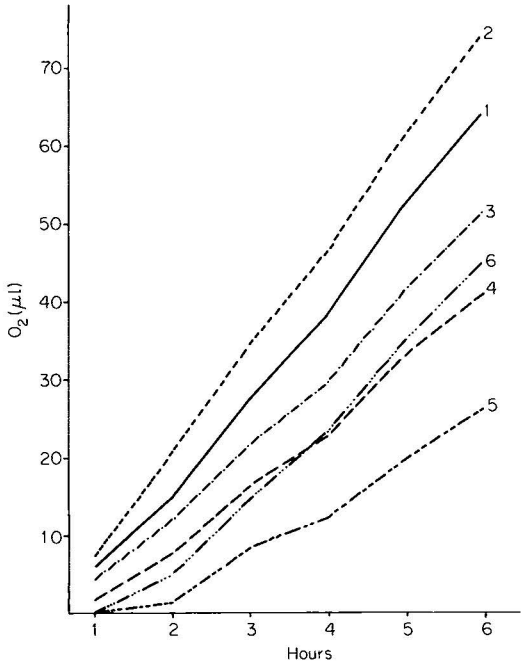

FIG. 7. Warburg respiration of *Boletus granulatus* cells with 20% RI2 metabolite (No. 1), 10% (No. 2), 5% (No. 3), 2% (No. 4), 1% (No. 5), and 0% (No. 6).

and nonmycorrhizal root tips. There is little doubt, that if diffusion occurs, these substances certainly exert some and perhaps considerable action on the rhizospheric microflora (Fig. 8).

2. *Influence of Rhizospheric Microflora on Symbiont Growth*

Despite the fact that all the literature on this subject was not examined, it is apparent that it has not received the attention of many researchers. There is no lack of research on the metabolism of rhizospheric microorganisms, especially on transformation of the soil's organic substance and their influence on plant development (Clark, 1949; Gerretsen, 1948; Katznelson and Bose, 1959; Katznelson *et al.*, 1948; Krasilnikov and Kotelev, 1956; Louw and Webley, 1959; Levishon, 1952; Pantos, 1956; Rovira and Bowen, 1960; Sperber, 1958; Starkey, 1958; Timonin, 1946; McCalla and Haskins, 1964; Norman, 1959; Parkinson, 1967; Rovira, 1965b; Rovira and Bowen, 1966), but studies on the direct influence of the rhizospheric microflora, or terrestrial microflora, in general, on the

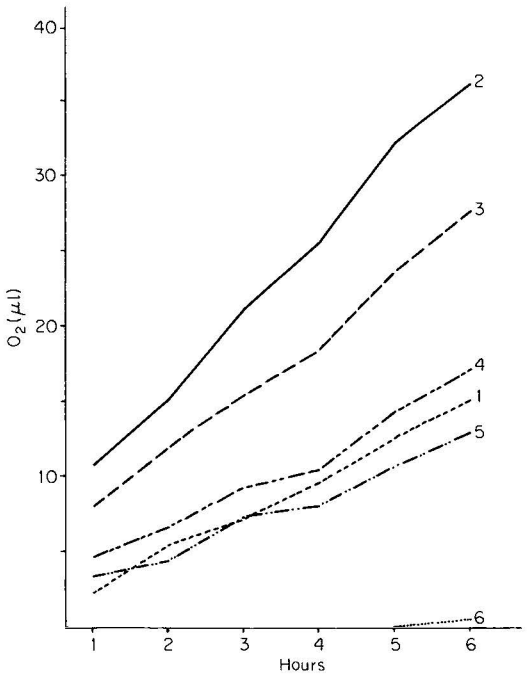

FIG. 8. Warburg respiration of *Boletus granulatus* cells in medium without nitrogen and with the following percentages of metabolite produced by RI2: 20% (No. 1), 10% (No. 2), 5% (No. 3), 2% (No. 4), 1% (No. 5), and 0% (No. 6).

plant symbiont or symbionts are very scarce. This influence, nevertheless, cannot be denied. Wilde as far back as in 1954 stated: "It appears highly probable that in grassland soils the survival of mycorrhizal fungi without their woody symbionts is precluded by toxic and antibiotic substances emitted by roots of prairie plants." But certainly, it is not only the toxic substances released by herbaceous plants that influence the viability of the symbionts. Without doubt great importance should also be attached to all the interactions of antagonism and competition that normally occur in the soil. The mechanisms described above create a special microbial ambient of special significance around the mycorrhizal root tips. These microorganisms associated with the mycorrhizae have an important task in maintaining symbiosis and survival of the fungus. This is supported by observations that in many environments the symbiont dies when the young abundantly mycorrhizal seedlings of *Pinus radiata* are transplanted. Undoubtedly many factors are involved in this phenomenon, but the contribution made by the rhizospheric microflora to the stability of the mycorrhizal association should not be underestimated.

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